

SUPPORT FOR THE AMENDMENTS

The specification has been amended to remove the hyperlink at page 17.

The sequence listing has been replaced. The sequence listing contains the human CK2  $\alpha$ ,  $\alpha'$  and  $\beta$  subunit sequences have the accession numbers NM\_007787, NM\_001896 and NM\_001320, respectively, as of the priority date for the present application. See Annexes 1-3, which show the sequences present in these records as of the priority date.

The amendments to the claims and newly-added Claim 27 are supported by the specification and the original claims.

No new matter is believed to have been added to the present application by the amendments submitted above.

REMARKS

Claims 1-25 and 27 are pending. Favorable reconsideration is respectfully requested.

The present invention relates to a double-stranded oligonucleotide comprising two strands of 19 to 23 nucleotides, each strand consisting, from 5' to 3', of a sequence of 17 to 21 ribonucleotides and two deoxyribo- or ribonucleotides, the 17 to 21 ribonucleotide RNA sequences of said strands being complementary and the two nucleotides of the 3' ends being protruding, wherein the RNA sequence of the sense strand or positive strand is that of a fragment of a transcript of an  $\alpha$ ,  $\alpha'$  or  $\beta$  subunit of a CK2 protein kinase, selected from the group consisting of:

- a) a fragment of a transcript of an  $\alpha$  subunit included between positions 18-74, 259-279, 565-585, 644-664, 720-750, 808-831 and 863-885, from the ATG codon, with reference to the sequence SEQ ID NO: 88,
- b) a fragment of a transcript of an  $\alpha'$  subunit included between positions 49-69, 132-142, 306-326, 367-387, 427-447, 451-471, 595-615, 735-755, 827-847, 868-888, 949-969 and 988-1008, from the ATG codon, with reference to the sequence SEQ ID NO: 89,
- c) a fragment of a transcript of a  $\beta$  subunit included between positions 80-100, 116-127, 164-208, 369-389, 400-420, 527-591 and 613-643, from the ATG codon, with reference to the sequence SEQ ID NO: 90, and
- d) a fragment of 17 to 21 bases exhibiting at least 80% identity with the fragments defined in a), b), and c).

See Claim 1.

Regarding the Restriction Requirement, Applicants submit that Claims 6 and 8 are part of the elected invention, since they are directed to elected nucleotide sequence SEQ ID NO: 26. SEQ ID NO: 67 (Claim 6) is directed to the elected nucleotide sequence SEQ ID NO: 26, as shown in Table III.

Regarding the IDS, the Buchou et al. reference was listed as category A in the International Search Report.

The hyperlink has been removed on page 17.

The objection to Claim 21 is believed to be obviated by the amendment submitted above. Withdrawal of the same is respectfully requested.

The rejection of the claims under 35 U.S.C. §112, second paragraph, is believed to be obviated by the amendment submitted above. Accordingly, Applicants submit that the claims are definite within the meaning of 35 U.S.C. §112, second paragraph.

The rejection of the claims under 35 U.S.C. §102(e) over Wyatt et al. (U.S. 6,440,738) is respectfully traversed.

The double-stranded oligonucleotide of Claim 1 is a siRNA composed of a 17 to 21 ribonucleotide double-stranded RNA sequence and two protruding nucleotides (ribonucleotides or deoxyribonucleotide) on both 3' ends. Therefore, the sense and antisense strands of the siRNA as claimed in Claims 9 and 11 comprise a 17 to 21 ribonucleotide RNA sequence.

Wyatt et al. disclose an antisense oligodeoxynucleotide composed of a central gap region consisting of ten 2'-deoxynucleotides, which is flanked on both sides by wings composed of five 2'-methoxyethyl (2'-MOE) nucleotides (column 46, lines 18-22).

The antisense oligonucleotide (ASO) of Wyatt et al. which is composed of 10 deoxynucleotides flanked on both sides with five modified oligonucleotides is different from the oligonucleotide of Claim 9 and 11 which is composed of 17 to 21 ribonucleotide flanked on the 3' end with 2 ribonucleotides or deoxyribonucleotides.

In view of the foregoing, the claimed oligonucleotide is not disclosed by Wyatt et al. Accordingly, the claims are not anticipated by that reference. Withdrawal of this ground of rejection is respectfully requested.

The rejections of the claims under 35 U.S.C. §103(a) over Wyatt et al. in view of Noonberg et al. and in view of Bass and Fosnaugh et al. and over John et al. in view of Fosnaugh et al. are respectfully traversed. The cited references fail to suggest the claimed oligonucleotide.

The siRNA of Claim 1 which target positions 18-74, 259-279, 565-585, 644-664, 720-750, 803-831 and 863-885 of the human CK2  $\alpha$  subunit transcript, positions 49-69, 132-142, 306-326, 367-387, 427-447, 451-471, 595-615, 735-755, 827-847, 868-888, 949-969 and 988-1008 of the human CK2  $\alpha$  subunit transcript and positions 80-100, 116-127, 164-208, 369-389, 400-420, 527-591 and 613-643 of the human CK2  $\beta$  subunit transcript, are not obvious to a person having ordinary skill in the art, for the following reasons.

Wyatt et al. teach several antisense oligodeoxynucleotides (ASO) targeted to the  $\beta$  subunit of the human CK2 protein (Table 1).

However, ASO and siRNA are totally different molecules that use totally different mechanisms to inhibit gene expression.

ASO consist of single-strand of 12-22 oligodeoxynucleotides which are complementary to the target mRNA sequence. Binding of the ASO to target mRNA results in steric inhibition of translation by ribosomal complex but more importantly stimulates degradation of the mRNA via RNase H.

siRNA consist of short RNA duplexes of 21-23 ribonucleotides which are incorporated into a ribonucleoprotein-endonuclease complex termed "*RNA Induced Silencing Complex*" (RISC). The siRNAs are then unwound and the antisense strand directs the complex to target the specific endogenous RNA sequence. The target RNA transcript is then bound and degraded by the endonuclease activity of RISC.

Therefore, there are fundamental differences in the sequence features which correlate with silencing efficacies for ASO and siRNA (see for example Z.J. Lu and D.H. Mathews, *Nucleic Acids Research*, 2008, 36, n° 11; *Annex 4*).

Thus, a person having ordinary skill in the art who is willing to make siRNA targeting the transcripts of the different CK2 subunits, would not find any teaching in Wyatt et al.

Such a person would not find any such teaching to make siRNA targeting the transcripts of the different CK2 subunits in Noonberg et al., Bass et al., Fosnaugh et al. and John et al. as well.

Using various luciferase transgenes as targets, Bass et al., teach only that the length of the siRNA is crucial to achieve sequence specific inhibition of gene expression in mammalian cells; *“the siRNAs have sense and antisense strands of about 21 nucleotides that form 19 base pairs to leave overhangs of two nucleotides at each 3' end”* (see figure 1). Furthermore, Bass et al. indicates that *“siRNAs are thought to provide the sequence information that allows a specific messenger RNA to be targeted for degradation”*. Therefore, it clearly emerges from Bass et al. that apart from the length of the siRNA, nothing is known about the sequence features of the siRNA which are capable of achieving specific inhibition of gene expression mammalian cells.

Fosnaugh et al., that relate to siRNA capable of inhibiting the expression of adenosine A1 receptor (ADORA1) and related receptors, provide only a list of 161 siRNAs targeted to overlapping sequences covering the entire length of the ADORA1 mRNA sequence (see Table I). Fosnaugh et al. does not show any gene silencing activity data with any of the 161 siRNAs presented. Therefore, a person having ordinary skill in the art could not deduce from this example relating to a particular gene (ADORA1), any general feature as regard the siRNA sequence or the mRNA target sequence that would help him to make siRNA targeting another gene (human CK2 subunits).

Noonberg et al. are totally silent about siRNA and teach only vectors for the intracellular delivery of antisense, triplex, and/or ribozyme oligonucleotides.

John et al. are totally silent about siRNA and teach only nanoparticles for the intracellular delivery of biologically active agents.

Furthermore, although many algorithms to design siRNAs were available at the time the invention was made, it was difficult to design siRNAs with high efficiency to its target. No solutions to this problem have been found so far. Today, it is difficult to design siRNAs with high efficiency to its target (see a recent report by W. Li and L. Cha, *Cell. Mol. Life Sci.*, 2007, 64, 1785-1792; *Annex 5*). These difficulties may be explained by the fact that there are many factors involved in siRNA efficiency (secondary structures of the sense and antisense strands of siRNAs, secondary structures of target mRNAs, sequence characteristics of siRNAs and the specificity of siRNA to its target). Current programs are mainly based on factors such as the secondary structure of siRNA antisense strands rather than comprehensive analysis of all factors.

In view of the foregoing, the claimed oligonucleotide is not suggested by Wyatt et al. in view of Noonberg et al. and in view of Bass and Fosnaugh et al. and over John et al. in view of Fosnaugh et al. are respectfully traversed. Accordingly, the claims are not obvious over those references. Withdrawal of these rejections are respectfully requested.

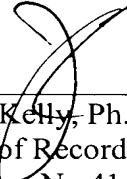
A paper copy of the substitute Sequence Listing and a computer-readable form thereof are enclosed. Applicants confirm that the sequence information in the paper copy of the substitute Sequence Listing and the computer-readable form thereof are the same.

Application No. 10/563,011  
Reply to Office Action of January 10, 2008

Applicants submit that the present application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,  
MAIER & NEUSTADT, P.C.




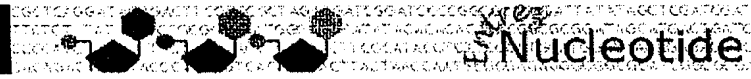
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(OSMMN 08/07)

Annex 1

[PubMed](#)
[Nucleotide](#)
[Protein](#)
[Genome](#)
[Structure](#)
[PMC](#)
[Taxonomy](#)
[OMIM](#)
[Books](#)

Search  Nucleotide  for:

Display    Hide: ☐ sequence ☐ all but gene, CDS and mRNA features

Range: from  to  ☐ Reverse complemented strand Features:

☐ 1: [NM\\_001895](#). Reports ...[gi:29570794] The record has been replaced by [NM\\_001895.3](#)

[Comment](#) [Features](#) [Sequence](#)

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 DEFINITION Homo sapiens casein kinase 2, alpha 1 polypeptide (CSNK2A1), transcript variant 2, mRNA.  
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 ORGANISM [Homo sapiens](#)  
 Eukaryota; Metazoa; Chordata; Crani ata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catar rhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 2323)  
 AUTHORS Faust,M., Gunther,J., Morgenstern,E ., Montenarh,M. and Gotz,C.  
 TITLE Specific localization of the cataly tic subunits of protein kinase CK2 at the centrosomes  
 JOURNAL Cell. Mol. Life Sci. 59 (12), 2155 -2164 (2002)  
 PUBMED [12568341](#)  
 REMARK GeneRIF: Data point to a particular role of the catalytic alpha and alpha' subunits of protein kinase C K2, which may be different from their roles in the holoenzyme.

REFERENCE 2 (bases 1 to 2323)  
 AUTHORS Skjerpen,C.S., Nilsen,T., Wesche,J. and Olsnes,S.  
 TITLE Binding of FGF -1 variants to protein kinase CK2 correlates wit h mitogenicity  
 JOURNAL EMBO J. 21 (15), 4058 -4069 (2002)  
 PUBMED [12145206](#)  
 REMARK GeneRIF: FGF -1 binds to both the catalytic alpha-subunit and to the regulatory beta -subunit of CK2. FGF-1 & CK2 alpha are shown to interact in vivo. A correlation bet ween the mitogenic potential of FGF-1 mutants & their ability to bind to CK2 alpha was observed.

REFERENCE 3 (bases 1 to 2323)  
 AUTHORS Sarno,S., Ghisellini,P. and Pinna,L .A.  
 TITLE Unique activation mechanism of prot ein kinase CK2. The N-terminal segment is essential for constituti ve activity of the catalytic subunit but not of the holoenzyme  
 JOURNAL J. Biol. Chem. 277 (25), 22509 -22514 (2002)  
 PUBMED [11956194](#)  
 REMARK GeneRIF: Unique activation mechanis m of protein kinase CK2. The N-terminal segment is essential for constitutive activity of the catalytic subunit but not of the ho loenzyme

REFERENCE 4 (bases 1 to 2323)  
 AUTHORS Messenger,M.M., Saulnier,R.B., Gilc hrist,A.D., Diamond,P., Gorbsky,G.J. and Litchfield,D.W.  
 TITLE Interactions between protein kinase CK2 and Pin1. Evidence for phosphorylation -dependent interactions  
 JOURNAL J. Biol. Chem. 277 (25), 23054 -23064 (2002)  
 PUBMED [11940573](#)  
 REMARK GeneRIF: Interactions between prote in kinase CK2 and Pin1. Evidence for phosphorylation -dependent interactions.

REFERENCE 5 (bases 1 to 2323)  
 AUTHORS Stepanova,V., Jerke,U., Sagach,V., Lindschau,C., Dietz,R., Haller,H. and Dumler,I.  
 TITLE Urokinase-dependent human vascular smooth muscle cell adh esion requires selective vitronectin phos phorylation by ectoprotein kinase CK2  
 JOURNAL J. Biol. Chem. 277 (12), 10265 -10272 (2002)  
 PUBMED [11756447](#)  
 REMARK GeneRIF: uPA -dependent VSMC adhesion is a function of select ive Vn phosphorylation by the ectoprotein kinase CK2



REFERENCE 6 (bases 1 to 2323)  
 AUTHORS Martel,V., Filhol,O., Nueda,A., Ger ber,D., Benitez,M.J. and Cochet,C.  
 TITLE Visualization and molecular analysi s of nuclear import of protein kinase CK2 subunits in living cells  
 JOURNAL Mol. Cell. Biochem. 227 (1 -2), 81-90 (2001)  
 PUBMED [11827178](#)

REFERENCE 7 (bases 1 to 2323)  
 AUTHORS Faust,M., Jung,M., Gunther,J., Zimm ermann,R. and Montenarh,M.  
 TITLE Localization of individual subunits of protein kinase CK2 to the endoplasmic reticulum and to the Go lgi apparatus  
 JOURNAL Mol. Cell. Biochem. 227 (1 -2), 73-80 (2001)  
 PUBMED [11827177](#)  
 REMARK GeneRIF: Localization of individual subunits of protein kinase CK2 to the endoplasmic reticulum and to the Golgi apparatus

REFERENCE 8 (bases 1 to 2323)  
 AUTHORS Yu,S., Wang,H., Davis,A. and Ahmed, K.  
 TITLE Consequences of CK2 signaling to th e nuclear matrix  
 JOURNAL Mol. Cell. Biochem. 227 (1 -2), 67-71 (2001)  
 PUBMED [11827176](#)  
 REMARK GeneRIF: Consequences of CK2 signal ing to the nuclear matrix.

REFERENCE 9 (bases 1 to 2323)  
 AUTHORS Pyerin,W. and Ackermann,K.  
 TITLE Transcriptional coordination of the genes encoding catalytic (CK2alpha) and regulatory (CK2beta) subunits of human protein kinase CK2  
 JOURNAL Mol. Cell. Biochem. 227 (1 -2), 45-57 (2001)  
 PUBMED [11827174](#)  
 REMARK GeneRIF: Transcriptional coordinati on of the genes encoding catalytic (CK2alpha) and regulatory (CK2beta) subunits of human protein kinase CK2.

REFERENCE 10 (bases 1 to 2323)  
 AUTHORS Guerra,B., Niefind,K., Ermakowa,I. and Issinger,O.G.  
 TITLE Characterization of CK2 holoenzyme variants with regard to crystallization  
 JOURNAL Mol. Cell. Biochem. 227 (1 -2), 3-11 (2001)  
 PUBMED [11827171](#)  
 REMARK GeneRIF: Characterization of CK2 ho loenzyme variants with regard to crystallization

REFERENCE 11 (bases 1 to 2323)  
 AUTHORS Litchfield,D.W., Bosc,D.G., Canton, D.A., Saulnier,R.B., Vilks,G. and Zhang,C.  
 TITLE Functional specialization of CK2 is oforms and characterization of isoform-specific binding partners  
 JOURNAL Mol. Cell. Biochem. 227 (1 -2), 21-29 (2001)  
 PUBMED [11827170](#)  
 REMARK GeneRIF: Functional specialization of CK2 isoforms and characterization of isoform -specific binding partners

REFERENCE 12 (bases 1 to 2323)  
 AUTHORS Wang,H., Davis,A., Yu,S. and Ahmed, K.  
 TITLE Response of cancer cells to molecu lar interruption of the CK2 signal  
 JOURNAL Mol. Cell. Biochem. 227 (1 -2), 167-174 (2001)  
 PUBMED [11827168](#)  
 REMARK GeneRIF: Response of cancer cells t o molecular interruption of the CK2 signal.

REFERENCE 13 (bases 1 to 2323)  
 AUTHORS Landesman-Bollag,E., Song,D.H., Romieu -Mourez,R., Sussman,D.J., Cardiff,R.D., Sonenshein,G.E. and S eldin,D.C.  
 TITLE Protein kinase CK2: signaling and t umorigenesis in the mammary gland  
 JOURNAL Mol. Cell. Biochem. 227 (1 -2), 153-165 (2001)  
 PUBMED [11827167](#)  
 REMARK GeneRIF: Protein kinase CK2: signal ing and tumorigenesis in the mammary gland.

REFERENCE 14 (bases 1 to 2323)  
 AUTHORS Meggio,F., Marin,O., Boschetti,M., Sarno,S. and Pinna,L.A.  
 TITLE HIV-1 Rev transactivator: a beta -subunit directed substrate and effector of protein kinase CK2  
 JOURNAL Mol. Cell. Biochem. 227 (1 -2), 145-151 (2001)  
 PUBMED [11827166](#)  
 REMARK GeneRIF: HIV -1 Rev transactivator: a beta-subunit directed substrate and effector of protein k inase CK2

## NCBI Sequence Viewer v2.0

Page 3 sur 5

REFERENCE 15 (bases 1 to 2323)  
 AUTHORS Sarno,S., Ghisellini,P., Cesaro,L., Battistutta,R. and Pinna,L.A.  
 TITLE Generation of mutants of CK2alpha which are dependent on the  
 beta-subunit for catalytic activity  
 JOURNAL Mol. Cell. Biochem. 227 (1 -2), 13-19 (2001)  
 PUBMED [11827164](#)  
 REMARK GeneRIF: Generation of mutants of C K2alpha which are dependent on  
 the beta-subunit for catalytic activity

REFERENCE 16 (bases 1 to 2323)  
 AUTHORS Bosc,D.G., Graham,K.C., Saulnier,R. B., Zhang,C., Prober,D.,  
 Gietz,R.D. and Litchfield,D.W.  
 TITLE Identification and characterization of CKIP-1, a novel pleckstrin  
 homology domain -containing protein that interacts with protein  
 kinase CK2  
 JOURNAL J. Biol. Chem. 275 (19), 14295 -14306 (2000)  
 PUBMED [10799509](#)

REFERENCE 17 (bases 1 to 2323)  
 AUTHORS Wirkner,U., Voss,H., Ansorge,W. and Pyerin,W.  
 TITLE Genomic organization and promoter identification of the human  
 protein kinase CK2 catalytic subunit alpha (CSNK2A1)  
 JOURNAL Genomics 48 (1), 71 -78 (1998)  
 PUBMED [9503018](#)

REFERENCE 18 (bases 1 to 2323)  
 AUTHORS Wirkner,U., Voss,H., Lichter,P., Ansorge,W. and Pyerin,W.  
 TITLE The human gene (CSNK2A1) coding for the casein kinase II subunit  
 alpha is located on chromosome 20 and contains tandemly arranged  
 Alu repeats  
 JOURNAL Genomics 19 (2), 257 -265 (1994)  
 PUBMED [8188256](#)

REFERENCE 19 (bases 1 to 2323)  
 AUTHORS Boldyreff,B., Klett,C., Gottert,E., Geurts van Kessel,A.,  
 Hameister,H. and Issinger,O.G.  
 TITLE Assignment of casein kinase 2 alpha sequences to two different  
 human chromosomes  
 JOURNAL Hum. Genet. 89 (1), 79 -82 (1992)  
 PUBMED [1577469](#)

REFERENCE 20 (bases 1 to 2323)  
 AUTHORS Yang-Feng,T.L., Zheng,K., Kopatz,I., Naiman,T. and C anaani,D.  
 TITLE Mapping of the human casein kinase II catalytic subunit genes: two  
 loci carrying the homologous sequences for the alpha subunit  
 JOURNAL Nucleic Acids Res. 19 (25), 7125 -7129 (1991)  
 PUBMED [1766873](#)

REFERENCE 21 (bases 1 to 2323)  
 AUTHORS Lozeman,F.J., Litchfield,D.W., Pien ing,C., Takio,K., Walsh,K.A. and  
 Krebs,E.G.  
 TITLE Isolation and characterization of human cDNA clones encoding the  
 alpha and the alpha' subunits of casein kinase II  
 JOURNAL Biochemistry 29 (36), 8436 -8447 (1990)  
 PUBMED [2174700](#)

REFERENCE 22 (bases 1 to 2323)  
 AUTHORS Meisner,H., Heller -Harrison,R., Buxton,J. and Czech,M.P.  
 TITLE Molecular cloning of the human casein kinase II alpha subunit  
 JOURNAL Biochemistry 28 (9), 4072 -4076 (1989)  
 PUBMED [2752008](#)

COMMENT REVIEWED REFSEQ: This record has been curated by NCBI staff. The  
 reference sequence was derived from [M55265.1](#) and [AF011920.1](#).  
 On Apr 7, 2003 this sequence version replaced gi: [4503094](#).

Summary: Casein kinase II is a serine/threonine protein kinase that  
 phosphorylates acidic proteins such as casein. The kinase exists as  
 a tetramer and is composed of an alpha, an alpha-prime, and two  
 beta subunits. The alpha subunits contain the catalytic activity  
 while the beta subunits undergo autophosphorylation. The protein  
 encoded by this gene represents the alpha subunit. While this gene  
 is found on chromosome 20, a related transcribed pseudogene is  
 found on chromosome 11. Three transcript variants encoding two  
 different isoforms have been found for this gene.

Transcript Variant: This variant (2) differs in the 5' UTR compared  
 to variant 1. Variants 1 and 2 both encode isoform a.  
 COMPLETENESS: complete on the 5' end.

FEATURES  
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Page 5 sur 5


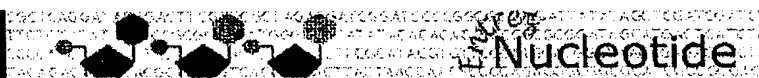
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Last update: Tue, 29 Apr 2008 Rev. 126150

Annex 2

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[Taxonomy](#)
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[Books](#)

Search  Nucleotide  for

Display     ☐ sequence ☐ all but gene, CDS and mRNA features

Range: from  begin  to  end ☐ Reverse complemented strand Features: ☐ SNP

☐ 1: [NM\\_001896](#). Reports ...[gi:4503096] The record has been replaced by [NM\\_001896.2](#)

[Comment](#) [Features](#) [Sequence](#)

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 REFERENCE 1 (bases 1 to 1677)  
 AUTHORS Faust,M., Gunther,J., Morgenstern,E., Montenarh,M. and Gotz,C.  
 TITLE Specific localization of the catalytic subunits of protein kinase CK2 at the centrosomes  
 JOURNAL Cell. Mol. Life Sci. 59 (12), 2155 -2164 (2002)  
 PUBMED [12568341](#)  
 REMARK GeneRIF: Data point to a particular role of the catalytic alpha and alpha' subunits of protein kinase C K2, which may be different from their roles in the holoenzyme.  
 REFERENCE 2 (bases 1 to 1677)  
 AUTHORS Skjerpen,C.S., Nilsen,T., Wesche,J. and Olsnes,S.  
 TITLE Binding of FGF -1 variants to protein kinase CK2 correlates with mitogenicity  
 JOURNAL EMBO J. 21 (15), 4058 -4069 (2002)  
 PUBMED [12145206](#)  
 REMARK GeneRIF: FGF -1 binds to both the catalytic alpha-subunit & to the regulatory beta -subunit of CK2. FGF-1 & CK2 alpha are shown to interact in vivo. A correlation between the mitogenic potential of FGF-1 mutants & their ability to bind to CK2 alpha was observed.  
 REFERENCE 3 (bases 1 to 1677)  
 AUTHORS Homma,M.K., Li,D., Krebs,E.G., Yuasa,Y. and Homma,Y.  
 TITLE Association and regulation of casein kinase 2 activity by adenomatous polyposis coli protein  
 JOURNAL Proc. Natl. Acad. Sci. U.S.A. 99 (9), 5959-5964 (2002)  
 PUBMED [11972058](#)  
 REMARK GeneRIF: Association and regulation of casein kinase 2 activity by adenomatous polyposis coli protein  
 REFERENCE 4 (bases 1 to 1677)  
 AUTHORS Faust,M., Jung,M., Gunther,J., Zimmermann,R. and Montenarh,M.  
 TITLE Localization of individual subunits of protein kinase CK2 to the endoplasmic reticulum and to the Golgi apparatus  
 JOURNAL Mol. Cell. Biochem. 227 (1-2), 73-80 (2001)  
 PUBMED [11827177](#)  
 REMARK GeneRIF: Localization of individual subunits of protein kinase CK2 to the endoplasmic reticulum and to the Golgi apparatus  
 REFERENCE 5 (bases 1 to 1677)  
 AUTHORS Litchfield,D.W., Bosco,D.G., Canton, D.A., Saulnier,R.B., Vilks,G. and Zhang,C.  
 TITLE Functional specialization of CK2 isoforms and characterization of isoform-specific binding partners  
 JOURNAL Mol. Cell. Biochem. 227 (1-2), 21-29 (2001)  
 PUBMED [11827170](#)  
 REMARK GeneRIF: Functional specialization of CK2 isoforms and characterization of isoform-specific binding partners  
 REFERENCE 6 (bases 1 to 1677)  
 AUTHORS Xu,X., Toselli,P.A., Russell,L.D. and Seldin,D.C.  
 TITLE Globozoospermia in mice lacking the casein kinase II alpha' catalytic subunit

## NCBI Sequence Viewer v2.0

Page 2 sur 3

JOURNAL Nat. Genet. 23 (1), 118 -121 (1999)  
 PUBMED [10471512](#)  
 REFERENCE 7 (bases 1 to 1677)  
 AUTHORS Yang-Feng,T.L., Naiman,T., Kopatz,I., Eli,D., Dafni, N. and Canaani,D.  
 TITLE Assignment of the human casein kinase II alpha' subunit gene (CSNK2A1) to chromosome 16p13.2 -p13.3  
 JOURNAL Genomics 19 (1), 173 (1994)  
 PUBMED [8188223](#)  
 REFERENCE 8 (bases 1 to 1677)  
 AUTHORS Lozeman,F.J., Litchfield,D.W., Piening,C., Takio,K., Walsh,K.A. and Krebs,E.G.  
 TITLE Isolation and characterization of human cDNA clones encoding the alpha and the alpha' subunits of casein kinase II  
 JOURNAL Biochemistry 29 (36), 8436 -8447 (1990)  
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Page 3 sur 3

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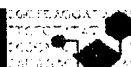

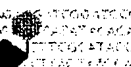
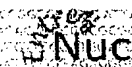
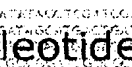
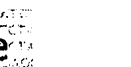

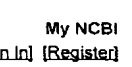
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[NCBI](#) | [NLM](#) | [NIH](#)

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Annex 3

Page 1 sur 4

Search:  for

Display:  Show:  Send to:  Hide: ☐ sequence ☐ all but gene, CDS and mRNA features

Range: from  to  ☐ Reverse complemented strand Features: ☐ SNP ☒ STS ☒ Exon

☐ 1: [NM\\_001320](#). Reports Homo sapiens case...[gi:26787971]

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[Links](#)
[Comment](#) [Features](#) [Sequence](#)

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REFERENCE 1 (bases 1 to 1128)  
 AUTHORS Skjerve, C.S., Nilsen, T., Wesche, J. and Olsnes, S.  
 TITLE Binding of FGF-1 variants to protein kinase CK2 correlates with mitogenicity  
 JOURNAL EMBO J. 21 (15), 4058-4069 (2002)  
 PUBMED [12145206](#)  
 REMARK GeneRIF: FGF-1 binds to both the catalytic alpha-subunit & to the regulatory beta-subunit of CK2. The presence of FGF-1 or FGF-2 was found to enhance the autophosphorylation of CK2 beta.

REFERENCE 2 (bases 1 to 1128)  
 AUTHORS Singh, L.S. and Kalafatis, M.  
 TITLE Sequencing of full-length cDNA encoding the alpha and beta subunits of human casein kinase II from human platelets and megakaryocytic cells. Expression of the casein kinase IIalpha intronless gene in a megakaryocytic cell line  
 JOURNAL Biochemistry 41 (28), 8935-8940 (2002)  
 PUBMED [12102635](#)  
 REMARK GeneRIF: sequencing of full-length DNA encoding subunits in platelets and megakaryocytic cells

REFERENCE 3 (bases 1 to 1128)  
 AUTHORS Homma, M.K., Li, D., Krebs, E.G., Yuasa, Y. and Homma, Y.  
 TITLE Association and regulation of casein kinase 2 activity by adenomatous polyposis coli protein  
 JOURNAL Proc. Natl. Acad. Sci. U.S.A. 99 (9), 5959-5964 (2002)  
 PUBMED [11972058](#)  
 REMARK GeneRIF: Association and regulation of casein kinase 2 activity by adenomatous polyposis coli protein

REFERENCE 4 (bases 1 to 1128)  
 AUTHORS Tchernev, V.T., Mansfield, T.A., Giot, L., Kumar, A.M., Nandabalan, K., Li, Y., Mishra, V.S., Detter, J.C., Rothberg, J.M., Wallace, M.R., Southwick, F.S. and Kingsmore, S.F.  
 TITLE The Chediak-Higashi protein interacts with SNARE complex and signal transduction proteins  
 JOURNAL Mol. Med. 8 (1), 56-64 (2002)  
 PUBMED [11984006](#)

REFERENCE 5 (bases 1 to 1128)  
 AUTHORS Martel, V., Filhol, O., Nueda, A., Gerber, D., Benitez, M.J. and Cochet, C.  
 TITLE Visualization and molecular analysis of nuclear import of protein kinase CK2 subunits in living cells  
 JOURNAL Mol. Cell. Biochem. 227 (1-2), 81-90 (2001)  
 PUBMED [11827178](#)

REFERENCE 6 (bases 1 to 1128)  
 AUTHORS Faust, M., Jung, M., Gunther, J., Zimmermann, R. and Montenarh, M.  
 TITLE Localization of individual subunits of protein kinase CK2 to the endoplasmic reticulum and to the Golgi apparatus  
 JOURNAL Mol. Cell. Biochem. 227 (1-2), 73-80 (2001)  
 PUBMED [11827177](#)  
 REMARK GeneRIF: Localization of individual subunits of protein kinase CK2 to the endoplasmic reticulum and to the Golgi apparatus

REFERENCE 7 (bases 1 to 1128)  
 AUTHORS Yu, S., Wang, H., Davis, A. and Ahmed, K.  
 TITLE Consequences of CK2 signaling to the nuclear matrix  
 JOURNAL Mol. Cell. Biochem. 227 (1-2), 67-71 (2001)  
 PUBMED [11827176](#)  
 REMARK GeneRIF: Consequences of CK2 signaling to the nuclear matrix.



## NCBI Sequence Viewer v2.0

Page 2 sur 4

- REFERENCE 8 (bases 1 to 1128)  
 AUTHORS Pyerin,W. and Ackermann,K.  
 TITLE Transcriptional coordination of the genes encoding catalytic (CK2alpha) and regulatory (CK2beta) subunits of human protein kinase CK2  
 JOURNAL Mol. Cell. Biochem. 227 (1-2), 45-57 (2001)  
 PUBMED [11827174](#)  
 REMARK GeneRIF: Transcriptional coordination of the genes encoding catalytic (CK2alpha) and regulatory (CK2beta) subunits of human protein kinase CK2.
- REFERENCE 9 (bases 1 to 1128)  
 AUTHORS Guerra,B., Niefind,K., Ermakowa,I. and Issinger,O.G.  
 TITLE Characterization of CK2 holoenzyme variants with regard to crystallization  
 JOURNAL Mol. Cell. Biochem. 227 (1-2), 3-11 (2001)  
 PUBMED [11827171](#)  
 REMARK GeneRIF: Characterization of CK2 holoenzyme variants with regard to crystallization
- REFERENCE 10 (bases 1 to 1128)  
 AUTHORS Wang,H., Davis,A., Yu,S. and Ahmed,K.  
 TITLE Response of cancer cells to molecular interruption of the CK2 signal  
 JOURNAL Mol. Cell. Biochem. 227 (1-2), 167-174 (2001)  
 PUBMED [11827168](#)  
 REMARK GeneRIF: Response of cancer cells to molecular interruption of the CK2 signal.
- REFERENCE 11 (bases 1 to 1128)  
 AUTHORS Meggio,F., Marin,O., Boschetti,M., Sarno,S. and Pinna,L.A.  
 TITLE HIV-1 Rev transactivator: a beta-subunit directed substrate and effector of protein kinase CK2  
 JOURNAL Mol. Cell. Biochem. 227 (1-2), 145-151 (2001)  
 PUBMED [11827166](#)  
 REMARK GeneRIF: HIV-1 Rev transactivator: a beta-subunit directed substrate and effector of protein kinase CK2
- REFERENCE 12 (bases 1 to 1128)  
 AUTHORS Ahn,B.H., Kim,T.H. and Bae,Y.S.  
 TITLE Mapping of the interaction domain of the protein kinase CKII beta subunit with target proteins  
 JOURNAL Mol. Cells 12 (2), 158-163 (2001)  
 PUBMED [11710515](#)  
 REMARK GeneRIF: Mapping of the interaction domain of the protein kinase CKII beta subunit with target proteins.
- REFERENCE 13 (bases 1 to 1128)  
 AUTHORS Keller,D.M., Zeng,X., Wang,Y., Zhang,Q.H., Kapoor,M., Shu,H., Goodman,R., Lozano,G., Zhao,Y. and Lu,H.  
 TITLE A DNA damage-induced p53 serine 392 kinase complex contains CK2, hSpt16, and SSRP1  
 JOURNAL Mol. Cell 7 (2), 283-292 (2001)  
 PUBMED [11239457](#)
- REFERENCE 14 (bases 1 to 1128)  
 AUTHORS Mucher,G., Becker,J., Knapp,M., Buttner,R., Moser,M., Rudnik-Schoneborn,S., Somlo,S., Germino,G., Onuchic,L., Avner,E., Guay-Woodford,L. and Zerres,K.  
 TITLE Fine mapping of the autosomal recessive polycystic kidney disease locus (PKHD1) and the genes MUT, RDS, CSNK2 beta, and GSTA1 at 6p21.1-p12  
 JOURNAL Genomics 48 (1), 40-45 (1998)  
 PUBMED [9503014](#)
- REFERENCE 15 (bases 1 to 1128)  
 AUTHORS Albertella,M.R., Jones,H., Thomson,W., Olavesen,M.G. and Campbell,R.D.  
 TITLE Localization of eight additional genes in the human major histocompatibility complex, including the gene encoding the casein kinase II beta subunit (CSNK2B)  
 JOURNAL Genomics 36 (2), 240-251 (1996)  
 PUBMED [8812450](#)
- REFERENCE 16 (bases 1 to 1128)  
 AUTHORS Pyerin,W.  
 TITLE Human casein kinase II: structures, genes, expression and requirement in cell growth stimulation  
 JOURNAL Adv. Enzyme Regul. 34, 225-246 (1994)  
 PUBMED [7942276](#)
- REFERENCE 17 (bases 1 to 1128)  
 AUTHORS Voss,H., Wirkner,U., Jakobi,R., Hewitt,N.A., Schwager,C., Zimmermann,J., Ansorge,W. and Pyerin,W.  
 TITLE Structure of the gene encoding human casein kinase II subunit beta  
 JOURNAL J. Biol. Chem. 266 (21), 13706-13711 (1991)  
 PUBMED [1856204](#)
- REFERENCE 18 (bases 1 to 1128)  
 AUTHORS Yang-Feng,T.L., Teltz,T., Cheung,M.C., Kan,Y.W. and Canaani,D.  
 TITLE Assignment of the human casein kinase II beta-subunit gene to

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Page 3 sur 4

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 JOURNAL Genomics 8 (4), 741-742 (1990)  
 PUBMED [2276748](#)  
 REFERENCE 19 (bases 1 to 1128)  
 AUTHORS Heller-Harrison,R.A., Meisner,H. and Czech,M.P.  
 TITLE Cloning and characterization of a cDNA encoding the beta subunit of human casein kinase II  
 JOURNAL Biochemistry 28 (23), 9053-9058 (1989)  
 PUBMED [2513884](#)  
 REFERENCE 20 (bases 1 to 1128)  
 AUTHORS Jakobi,R., Voss,H. and Pyerin,W.  
 TITLE Human phosvitin/casein kinase type II. Molecular cloning and sequencing of full-length cDNA encoding subunit beta  
 JOURNAL Eur. J. Biochem. 183 (1), 227-233 (1989)  
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 On Dec 13, 2002 this sequence version replaced [gi:23503294](#).

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Annex 4

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# Fundamental differences in the equilibrium considerations for siRNA and antisense oligodeoxynucleotide design

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## ABSTRACT

Both siRNA and antisense oligodeoxynucleotides (ODNs) inhibit the expression of a complementary gene. In this study, fundamental differences in the considerations for RNA interference and antisense ODNs are reported. In siRNA and antisense ODN databases, positive correlations are observed between the cost to open the mRNA target self-structure and the stability of the duplex to be formed, meaning the sites along the mRNA target with highest potential to form strong duplexes with antisense strands also have the greatest tendency to be involved in pre-existing structure. Efficient siRNA have less stable siRNA–target duplex stability than inefficient siRNA, but the opposite is true for antisense ODNs. It is, therefore, more difficult to avoid target self-structure in antisense ODN design. Self-structure stabilities of oligonucleotide and target correlate to the silencing efficacy of siRNA. Oligonucleotide self-structure correlations to efficacy of antisense ODNs, conversely, are insignificant. Furthermore, self-structure in the target appears to correlate with antisense ODN efficacy, but such that more effective antisense ODNs appear to target mRNA regions with greater self-structure. Therefore, different criteria are suggested for the design of efficient siRNA and antisense ODNs and the design of antisense ODNs is more challenging.

## INTRODUCTION

Antisense oligonucleotides, such as siRNA or antisense oligodeoxynucleotides (ODNs), can silence gene expression (1). siRNA associate with the protein–RNA complex called the RNA-induced silencing complex (RISC) to cleave the target mRNA or attenuate the gene expression with the RNAi pathway (2–4). Antisense ODNs also bind

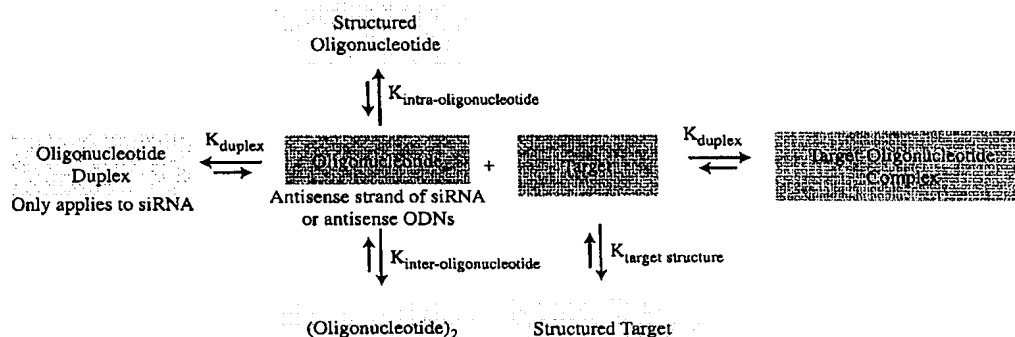
to a complementary region of the target mRNA and generally inhibit expression by stimulating degradation of the mRNA via RNase H (5–7).

The silencing efficacies of RNAi and antisense ODNs are found to correlate with their sequence features. Efficient siRNA have preference for low G/C content, A at position 3, U at position 10, absence of G at position 13, absence of G or C at position 19, etc. (8–14). Antisense ODN silencing efficacy also correlates highly with some specific motifs of oligonucleotide sequence, such as CCAC and ACTG (15,16). Additionally, the local secondary structure of the target mRNA also influences the binding affinity of siRNA (17–20) and antisense ODNs (21–24).

In this study, predicted free energy changes of hybridization of both antisense ODNs and siRNA are compared to inhibition efficacy databases to demonstrate contrasts in the hybridization terms that influence efficacy. Free energy changes of hybridization of the antisense oligonucleotide to the mRNA target are calculated using the OligoWalk algorithm (25,26), which uses the equilibrium shown in Figure 1. The equilibrium includes self-structure terms,  $\Delta G_{\text{intraoligonucleotide}}^{\circ}$ ,  $\Delta G_{\text{interoligonucleotide}}^{\circ}$  and  $\Delta G_{\text{target structure}}^{\circ}$ , which correspond to the free energy change of opening intramolecular pairs in the oligonucleotide, intermolecular pairs in the oligonucleotide and base pairs in the hybridization region of the target, respectively.

The stability of duplex hybridization between antisense sequence and target is found, for the first time, to be significantly correlated with the stability of the target mRNA's self-structure at the hybridization region for both siRNA and antisense ODNs. Duplex stability is also shown to be correlated with the oligonucleotide self-structure stability for both siRNA and antisense ODNs. Different preferences of duplex stability, however, are observed for siRNA and antisense ODNs. Because RNAi is attenuated by the unwinding cost of opening the siRNA duplex, efficient siRNA (or miRNA) usually have less stable sense–antisense duplexes (27). This is just the opposite for efficient antisense ODNs, where tight

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**Figure 1.** Equilibrium considered in the OligoWalk algorithm (25,26) for siRNA and antisense ODNs. The equilibrium constants,  $K_{\text{duplex}}$ ,  $K_{\text{target structure}}$ ,  $K_{\text{intraoligonucleotide}}$ , and  $K_{\text{interoligonucleotide}}$  are related to  $\Delta G_{\text{duplex}}^{\circ}$ ,  $\Delta G_{\text{target structure}}^{\circ}$ ,  $\Delta G_{\text{intraoligonucleotide}}^{\circ}$ , and  $\Delta G_{\text{interoligonucleotide}}^{\circ}$  by  $\Delta G^{\circ} = -RT \ln K$ , respectively. Self-folding in the target and self-structure in the oligonucleotide both compete with the formation of the oligonucleotide–target complex. Only RNA secondary structure interactions are considered in the calculations. The longer arrow for each equilibrium shows the generally favored direction of the equilibrium, i.e. a negative folding free energy change is predicted for an equilibrium favoring the direction of the longer arrow.

hybridization to the target is apparently required. Furthermore, in addition to duplex stability, siRNA silencing efficacy also significantly correlates with other terms such as the self-structure stabilities of siRNA and target mRNA. These correlations are not as strong for antisense ODN efficacy.

## MATERIALS AND METHODS

### Prediction of self-structure of oligonucleotide and target

To quantify the accessibility of oligonucleotide and target mRNA for hybridization, a free energy change of self-structure is predicted for opening base pairs in the region of complementarity to the target. A partition function ( $Q$ ) calculation (28,29) is used to predict the ensemble free energy change (26). For example, the free energy cost of opening the self-structure of a target binding site is calculated using:

$$\Delta G_{\text{target structure}}^{\circ} = -RT \ln \left( \frac{Q_{\text{unconstrained}}}{Q_{\text{constrained}}} \right)$$

where  $Q$  is a partition function that sums the equilibrium constants for all possible structures,  $s$ .

$$Q = \sum_s e^{-\Delta G(s)/RT}$$

$R$  is gas constant,  $T$  is absolute temperature, which was set to 310.15 K in this study,  $Q_{\text{unconstrained}}$  is the partition function of the native target structures,  $Q_{\text{constrained}}$  is the partition function of the target structures in the state where the oligonucleotide is able to bind. To predict the constrained partition function, the calculation is performed with a constraint that nucleotides in the binding region are forced single stranded. In order to reduce calculation time,  $\Delta G_{\text{target structure}}^{\circ}$  is calculated with a partition function of the local structure on mRNA binding site, i.e. only a certain number of nucleotides centered at the binding region (800 nt) is folded (26). It was previously

demonstrated that local folding of 800nt does not significantly affect the accuracy of the accessibility prediction (26). If the binding site is located close to the 5' or 3' end of the target, the same size of region is folded beginning from the end of the sequence, which means the binding site is not centered on the folding region.

For the oligonucleotide, all self-structure must be broken during duplex formation with the target, so the self-structure free energy change is predicted with:

$$\Delta G_{\text{oligonucleotide self structure}}^{\circ} = -RT \ln(Q)$$

Both unimolecular and bimolecular self structure are considered for the oligonucleotide using appropriate partition functions (26).

### Thermodynamic parameters

Folding free energy changes for individual structures are predicted using nearest-neighbor models. For RNA structures, the nearest neighbor parameters from Turner and co-workers are used (30). For DNA structures, the nearest neighbor parameters for DNA from the RNAstructure program (30) are used. In the case of ODN hybridization to RNA targets, DNA–RNA duplex parameters are used for helix formation (31).

### Databases

The experimental data for gene silencing efficacy of oligonucleotides is derived from two databases. One is derived from an antisense ODN database, AOBBase (32). 418 ODNs targeting 28 mRNA are used for this study. Thirty ODNs were removed from the original database because these sequences are not consistent in sequence with the Genbank database (33). The silencing efficacy of each oligonucleotide is represented as  $\ln(A)$ , the natural logarithm of Activity, which is defined as the ratio of gene expression after antisense silencing over the untreated control. For the correlation calculations, any value of activity that is  $<0$  is reset to 0.1% and any value that

Table 1. Correlations between  $\ln(A)^a$  and free energy change terms for both siRNA and Antisense ODNs

	siRNA		Antisense ODNs	
	<i>r</i>	<i>t</i> -test <i>P</i> -value <sup>c</sup>	<i>r</i>	<i>t</i> -test <i>P</i> -value <sup>c</sup>
$\ln(A) - \Delta G_{\text{duplex}}^{\circ}$	-0.250	$1.78 \times 10^{-15}$	0.160	0.001
$\ln(A) - \Delta G_{\text{target structure}}^{\circ}$	-0.197	$1.11 \times 10^{-15}$	0.141 (0.0798) <sup>d</sup>	0.004 (0.284) <sup>d</sup>
$\ln(A) - \Delta G_{\text{intra-oligonucleotide}}^{\circ}$	-0.186	$1.55 \times 10^{-15}$	-0.0653 (-0.212) <sup>d</sup>	0.183 ( $7.59 \times 10^{-7}$ ) <sup>d</sup>
$\ln(A) - \Delta G_{\text{inter-oligonucleotide}}^{\circ}$	-0.199	$3.33 \times 10^{-15}$	-0.0467 (-0.186) <sup>d</sup>	0.341 ( $1.45 \times 10^{-4}$ ) <sup>d</sup>
$\ln(A) - \Delta \Delta G_{\text{ends}}^{\circ}$	-0.351	$2.66 \times 10^{-15}$	0.0587	0.231
$\Delta G_{\text{duplex}}^{\circ} - \Delta G_{\text{target structure}}^{\circ}$	0.595	$2.22 \times 10^{-16}$	0.510	$4.44 \times 10^{-16}$
$\Delta G_{\text{duplex}}^{\circ} - \Delta G_{\text{intra-oligonucleotide}}^{\circ}$	0.524	$2.22 \times 10^{-16}$	0.103	0.035
$\Delta G_{\text{duplex}}^{\circ} - \Delta G_{\text{inter-oligonucleotide}}^{\circ}$	0.560	$<10^{-30}$	0.264	$4.48 \times 10^{-8}$
$\Delta G_{\text{duplex}}^{\circ} - \Delta \Delta G_{\text{ends}}^{\circ}$	0.0176	0.384	-0.0494	0.314

The correlations were calculated within Novartis data set (34) for siRNA and AOBASE data set for antisense ODNs (32). *r* is the correlation coefficient. The definition of each free energy term is provided in the Introduction and in Figure 1.

<sup>a</sup> $\ln(A)$  is the natural logarithm of Activity, which is the fraction of the targeted mRNA expression after gene silencing compared to the control. Negative correlations indicate that decreasing each folding free energy change (increased stability) results in increased  $\ln(\text{Activity})$  (decreased silencing efficacy).

<sup>b</sup>The values were calculated with the partition function method with folding size as 800 nucleotides centered on the binding site.

<sup>c</sup>A *P*-value (probability) below 0.05 is statistically significant (significant values are shown in bold).

<sup>d</sup>The value in the parenthesis is the correlation coefficient for the oligonucleotides having  $\Delta G_{\text{duplex}}^{\circ} \leq -30$  kcal/mol.

is >100% is reset to 99.9%. Two hundred and fifteen antisense ODNs induced more than 50% gene silencing (silencing efficacy = 1 - Activity), 103 induced more than 70% and 30 induced more than 90%. The second database is an siRNA database of experiments from Huesken *et al.* (34) at Novartis, which contains efficacy data for 2431 siRNAs targeting 31 mRNA sequences on random positions. Two thousand siRNAs have silencing efficacy >50%, 1222 of them have efficacy >70%, 369 have efficacy >90%. The silencing efficacies reported in the siRNA database are transformed to Activity (Activity = 1 - silencing efficacy) in order to calculate  $\ln(A)$ .

### Statistical analysis

Linear correlation coefficients (*r*) are calculated between the free energy changes of duplex formation and free energy changes for self-structure formation in both oligonucleotide and target mRNA. Correlations are also explored between  $\ln(\text{Activity})$  and thermodynamic features involved in the equilibrium of binding for both siRNA and antisense ODNs. The significance of each linear correlation (Table 1) is tested with a two-tailed *t*-test. The *t*-test is performed with the Statistics-Basic-0.42 Perl module downloaded from: <http://www.cpan.org> and the data analysis tool in Excel 2004 (Microsoft Inc). For this study, a *P*-value of the test <0.05 is considered to be a significant correlation, i.e. rejection of the null hypothesis that the correlation is by chance.

### RESULTS

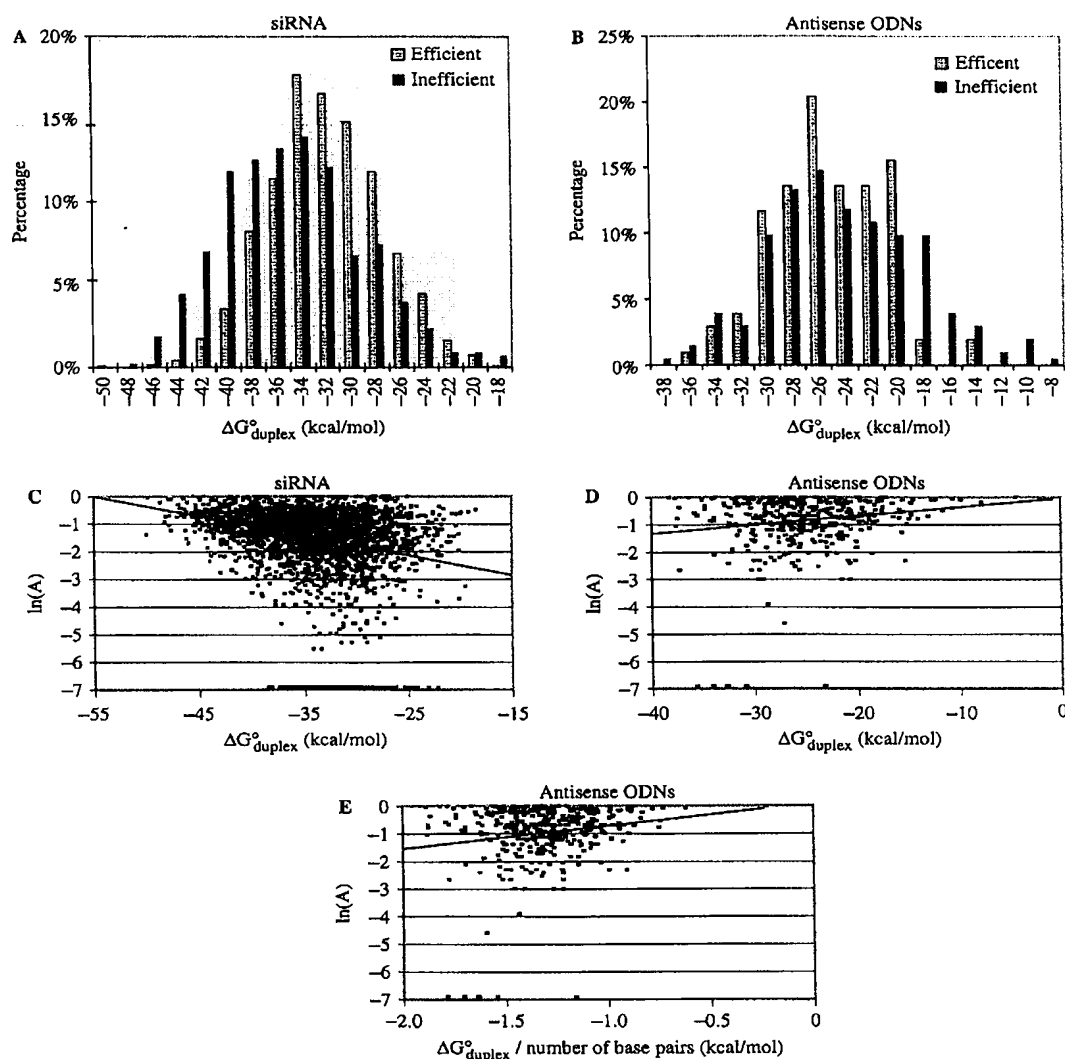
The OligoWalk algorithm (25,26) was developed to predict the affinity of a structured oligonucleotide to a structured RNA target using the equilibrium shown in Figure 1. The prediction explicitly considers self-structure of the oligonucleotide and target, quantified by

free energy changes calculated with the nearest neighbor model (30,31,35). The formation of self-structure ( $\Delta G_{\text{intraoligonucleotide}}^{\circ}$ ,  $\Delta G_{\text{interoligonucleotide}}^{\circ}$  and  $\Delta G_{\text{target structure}}^{\circ}$ ) competes with the hybridization of the antisense oligonucleotide to the target, which is driven by the favorable free energy change of duplex formation ( $\Delta G_{\text{duplex}}^{\circ}$ ). In addition, the stability difference between the duplex's two ends ( $\Delta \Delta G_{\text{ends}}^{\circ}$ ) was also calculated because it is well known that efficient siRNA prefer a less stable duplex at the 5' end of the antisense strand (8).

### Duplex stability requirements are different for siRNA and antisense ODNs

In RNAi, the siRNA duplex needs to unwind for loading the antisense strand on RISC and the antisense-target duplex needs to unwind for multiple turnover. Therefore, a general rule of siRNA design is a requirement for a low G/C content in the oligonucleotide (12). It was also reported that sense-antisense duplexes of efficient siRNA (or miRNA) are less stable than inefficient siRNA in previous studies (27,36). In this study, the same trend was observed in the Novartis siRNA database (34) (Figure 2A). The average  $\Delta G_{\text{duplex}}^{\circ}$  ( $-33.0 \pm 4.6$  kcal/mol) of efficient siRNA (silencing efficacy is not <70%) is 2.8 kcal/mol more than the average  $\Delta G_{\text{duplex}}^{\circ}$  ( $-35.8 \pm 5.7$  kcal/mol) of inefficient siRNA (silencing efficacy is <50%). Antisense ODNs, however, do not have to destabilize the duplex formation to be efficient and, in contrast to siRNA, require stable binding to the target (Figure 2B). The difference between the average  $\Delta G_{\text{duplex}}^{\circ}$  of efficient ODNs ( $-26.1 \pm 4.2$  kcal/mol) and inefficient ODNs ( $-24.9 \pm 5.9$  kcal/mol) is -1.2 kcal/mol.

The  $\ln(A)$ , natural logarithm of message activity, is plotted versus duplex free energy changes ( $\Delta G_{\text{duplex}}^{\circ}$ ) of all binding sites for siRNA and antisense ODNs, in Figure 2C and D, respectively. The correlation coefficient of  $\Delta G_{\text{duplex}}^{\circ}$  and  $\ln(A)$  is negative ( $r = -0.250$ ) for siRNA, yet positive ( $r = 0.160$ ) for antisense ODNs (Table 1).



**Figure 2.** Oligonucleotide–target duplex stabilities in siRNA and antisense ODNs databases. The histograms of free energy changes of oligonucleotide–target duplexes ( $\Delta G_{\text{duplex}}^{\circ}$ ) for efficient oligonucleotides (silencing efficacy is not <70%) and inefficient oligonucleotides (silencing efficacy is <50%) are shown in (A), the siRNA data set (34) and (B) the antisense ODNs data set (32). The duplex free energy change ( $\Delta G_{\text{duplex}}^{\circ}$ ) is plotted against  $\ln(A)$  for the siRNA database in (C) and the antisense ODNs database in (D). In (E),  $\ln(A)$  is plotted as a function of the per base pair duplex free energy change for the ODNs database.  $\ln(A)$  is the natural logarithm of Activity, which is the fraction of the targeted mRNA expression after antisense silencing compared to the control.

This shows again that less stable duplex formation is preferred by efficient siRNA but more stable duplexes are preferred by efficient antisense ODNs. Because the ODNs range in length from 9 to 21 nt, the correlation was also tested for  $\ln(A)$  as a function of ODN duplex free energy change per base pair (Figure 2E). The correlation coefficient is 0.181, with a  $P$ -value of 0.000207. This is an even stronger correlation than that between  $\ln(A)$  and  $\Delta G_{\text{duplex}}^{\circ}$ , which suggests that it is more important for antisense ODN activity to have stronger pairing per base pair than to simply favor longer helices.

#### Effect of self-structure appears different for siRNA and antisense ODNs

The silencing efficacy by siRNA has been previously demonstrated to be influenced by the secondary structures of both the antisense oligonucleotide and target mRNA (19,37). Each of the thermodynamic features calculated by OligoWalk,  $\Delta G_{\text{intraoligonucleotide}}^{\circ}$ ,  $\Delta G_{\text{interoligonucleotide}}^{\circ}$ ,  $\Delta G_{\text{target structure}}^{\circ}$ ,  $\Delta G_{\text{duplex}}^{\circ}$ , and  $\Delta \Delta G_{\text{ends}}^{\circ}$ , were previously shown to correlate with the gene-silencing efficacy by siRNA (26) (Table 1).

In this study, the same terms were calculated for 418 antisense ODNs with reported inhibition activities (32,38). Significant correlations were also found between  $\ln(A)$  and both  $\Delta G_{\text{target structure}}^{\circ}$  and  $\Delta G_{\text{duplex}}^{\circ}$  (Table 1). The correlation between  $\ln(A)$  and  $\Delta G_{\text{target structure}}^{\circ}$  is 0.141, which means that the more efficient antisense ODNs apparently anneal to regions of mRNA with more stable self-structure to be disrupted. This correlation is exactly opposite that for siRNAs and is counter-intuitive.

Furthermore, in contrast to siRNA, no significant correlations were observed between the free energy changes of oligonucleotide self-structure and the silencing efficacy of antisense ODNs. This is probably simply because of the wide range of ODN lengths. The correlations between oligonucleotide self-structure and  $\ln(A)$  can be improved using a  $\Delta G_{\text{duplex}}^{\circ}$  cutoff, where only sequences having  $\Delta G_{\text{duplex}}^{\circ} \leq -30$  kcal/mol are considered. After the cutoff, the lengths of the remaining antisense ODNs vary less, as most of them have 20 or 21 nt. For this subset of antisense ODNs, the antisense efficacy is statistically significantly influenced by the self-structure of oligonucleotide (Table 1). This is consistent with previous findings for antisense ODNs (39).

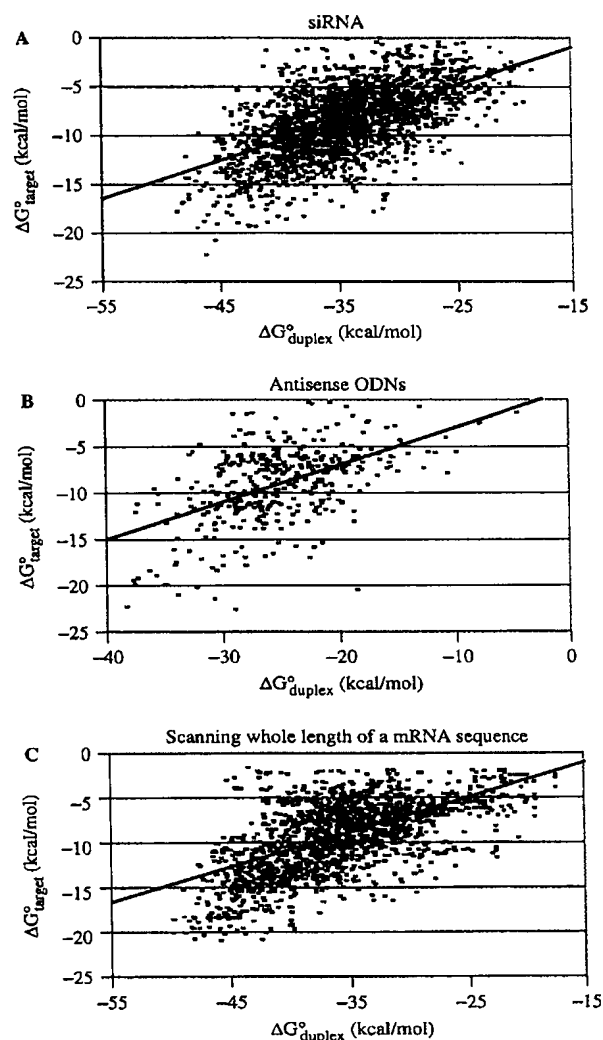
In a previous study of antisense ODNs (39), the self-structure of target was poorly predicted by either optimal or suboptimal structure prediction, which are not as rigorous as the partition function calculation used here. The ambiguous correlation between antisense efficacy and  $\Delta G_{\text{target structure}}^{\circ}$  in previous studies also comes from the relationship of hybridized duplex stability and self-structure accessibility of target (below).

#### Correlation between hybridized duplex stability and self-structure accessibility

To understand the basis of the different influence of target self-structure on siRNA and antisense ODNs, the relationship between  $\Delta G_{\text{duplex}}^{\circ}$  and the self-structure folding free energy changes was explored (Figure 3A and B). It was found that the duplex free energy change correlates significantly with each of the self-structure folding free energy changes ( $\Delta G_{\text{intraoligonucleotide}}^{\circ}$ ,  $\Delta G_{\text{interoligonucleotide}}^{\circ}$  and  $\Delta G_{\text{target structure}}^{\circ}$ ) for oligonucleotides in both the siRNA and antisense ODN database (Table 1). This correlation indicates that sequences that form stronger duplexes also tend to have stronger self-structures, both for the antisense sequences and for the target mRNA.

To control for whether this correlation is a result of a selection bias in the design of antisense sequences in the databases, it was tested for all 19mer antisense sequences in a complete scan of an mRNA (Genbank ID: X61940, length: 1933 bases) (Figure 3C). Again, the duplex-binding stability significantly correlates with the cost of opening the local self-structure of the target.

These correlations explain the apparent correlation that efficient antisense ODNs preferentially hybridize to targets with stronger self-structure. The strong correlation between target self-structure and duplex stability suggests the true preference for reduced target self-structure is obscured for ODNs because of the strong requirement for greater duplex stability. For siRNA, the correlation is



**Figure 3.** Correlations between free energy change of hybridized duplex ( $\Delta G_{\text{duplex}}^{\circ}$ ) and free energy cost of opening target base pairs for hybridization ( $\Delta G_{\text{target structure}}^{\circ}$ ). The  $\Delta G_{\text{target structure}}^{\circ}$  values were calculated with a partition function with a folding size of 800 nt centered on the binding site. (A) For the siRNA data set (34), the correlation coefficient is 0.5946 and the *t*-test *P*-value is  $2.22 \times 10^{-16}$ . (B) For the antisense ODNs data set (32), the correlation coefficient is 0.5097 and the *t*-test *P*-value is  $4.44 \times 10^{-16}$ . (C) For a full scan of an mRNA sequence (Genbank ID: X61940, length: 1933 bases) from the 5' end to 3' end, the correlation coefficient is 0.6187 and the *t*-test *P*-value is  $3.95 \times 10^{-30}$ .

readily observed because the requirement for reduced stability in the duplex also leads to a tendency for less target self-structure.

#### Differing equilibria for RNAi and antisense ODNs

In the initial step of RNA interference, the siRNA duplex needs to unwind (Figure 1), so the equilibrium constant



in the direction of the necessary product is  $1/K_{\text{duplex}}$ . Subsequently, the antisense strand hybridizes to mRNA, with the equilibrium constant for product of  $K_{\text{duplex}}$ . The cost of opening the mRNA self-structure is  $1/K_{\text{target structure}}$ . The overall equilibrium, including these three effects, relates to the log of activity:

$$\ln(A) \propto K_{\text{duplex}} \left( \frac{1}{K_{\text{duplex}}} \right) \left( \frac{1}{K_{\text{target structure}}} \right)$$

Because of the positive correlation between the hybridized duplex's stability ( $\Delta G_{\text{duplex}}^{\circ}$ ,  $K_{\text{duplex}}$ ) and the target structure's accessibility ( $\Delta G_{\text{target structure}}^{\circ}$ ,  $K_{\text{target structure}}$ ), the proportionality is then:

$$\ln(A) \propto \frac{1}{K_{\text{target structure}}} \propto \frac{1}{K_{\text{duplex}}}.$$

This suggests that siRNA design is simple because less stable duplexes target less stable target mRNA self-structures and efficient siRNA require both of these considerations at the same time.

For antisense ODNs, however, the opposite trend emerges because there is no duplex unwinding step involved in the inhibition mechanism. The cost of opening self-structure of target and oligonucleotide competes with the formation of hybridized duplex for antisense ODNs. When the self-structure thermodynamics are compared with  $\ln(A)$  for antisense ODNs, the self-structure stability correlates with the  $\ln(A)$  (Table 1), but in an unintuitive manner. The hybridized duplex stability apparently accounts the most for the efficacy of antisense ODNs. Therefore, in contrast to siRNA design, the requirement of stable duplex hybridization and unstable self-structure of target simultaneously makes design difficult.

## DISCUSSION

This study explores the underlying differences between the binding thermodynamics of RNAi and antisense ODNs. The preference of functional siRNA for low G/C content has been noted previously (27,40) and this leads to a lower stability for  $\Delta G_{\text{duplex}}^{\circ}$  (Figure 1). It is possible that the free energy cost to unwind the siRNA is more important than the stability of oligonucleotide–target duplex. This does not apply to antisense ODNs because antisense ODNs are delivered as a single-stranded agent. Another explanation is that turnover of RISC may be facilitated by having a lower duplex affinity between the siRNA and target. The cleavage mechanism of RISC has been well studied (41–44). RISC is an endonuclease that makes a single cleavage with preference to the middle of the mRNA binding site (10 nt from the 5' end of the siRNA) (41,45). The cleaved mRNA are released from RISC (41) and, presumably, the cleavage products are degraded in a common RNA degradation pathway because they do not have either the poly(A) tail or the 5' cap (45,46). The antisense siRNA in RISC is then intact for another round of cleavage (46). We speculate that it is possible that

RISC needs to open the base pairs between the siRNA and target mRNA strand in order to release the siRNA and RISC before degradation of the mRNA. This would lead to a preference for reduced binding strength by siRNA.

In contrast, functional antisense ODNs are known to prefer a stronger duplex affinity. In the antisense mechanism, RNase H binds to an RNA–DNA duplex and degrades the RNA. Although RNase H belongs to a nucleotidyl-transferase super family of enzymes that includes RISC (47), it may have a different process of cleavage. Experimental evidence suggests that RNase H degrades the RNA of a hybrid DNA–RNA duplex in a processive manner (48). The entire portion of the RNA strand in complex with the antisense ODN is probably degraded by RNase H and release of antisense ODN is facilitated regardless of the strength of antisense–target duplexes. Therefore, a propensity for strong duplex formation is important because it would favor target binding.

A number of studies have addressed the rational design of siRNA (12,34,49) and antisense ODNs (16,39,50), but these studies did not consider the structure features involved in the antisense binding using our rigorous partition function method. It has been demonstrated that including self-structure terms of siRNA and target mRNA helps the selection of efficient siRNA (26). The correlations found in this study show that different thermodynamic features could also be considered to improve the design of antisense ODNs. Contributions from multiple features of antisense ODNs need to be considered in order to find an optimized combination for an efficient candidate.

Another important factor in design of effective oligonucleotides is the accessibility of the target self-structure, which competes with the hybridization of the oligonucleotide to the target. The paradox demonstrated here is that the sequence features conducive to a stronger formation duplex also contribute to less binding accessibility because of self-structure of the target. This is observed as the positive correlation between the free energy changes of duplex formation and self-structure. Because siRNA favors less stable duplexes, it is easy to simultaneously avoid target structure in siRNA design. For antisense ODNs design, however, it is difficult to design strong duplexes that will bind to regions with little self-structure. This means it is fundamentally more difficult to design antisense ODNs than siRNA.

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Annex 5

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**Cellular and Molecular Life Sciences**

## Predicting siRNA efficiency

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**Abstract.** Since the identification of RNA-mediated interference (RNAi) in 1998, RNAi has become an effective tool to inhibit gene expression. The inhibition mechanism is triggered by introducing a short interference double-stranded RNA (siRNA, 19–27 bp) into the cytoplasm, where the guide strand of siRNA (usually antisense strand) binds to its target messenger RNA and the expression of the target gene is blocked. RNAi has been widely applied in gene functional analysis, and as a potential therapeutic

strategy in viral diseases, drug target discovery, and cancer therapy. Among the factors which may compromise inhibition efficiency, how to design siRNAs with high efficiency and high specificity to its target gene is critical. Although many algorithms have been developed for this purpose, it is still difficult to design such siRNAs. In this review, we will briefly discuss prediction methods for siRNA efficiency and the problems of present approaches.

**Keywords.** RNAi, siRNA, algorithm, prediction.

### Introduction

RNA interference (RNAi) is an ancient mechanism for gene regulation which is mediated by short interfering doublestrand RNA (siRNA) [1–2]. The basic steps in knockdown expression of an siRNA target gene are as follows. First, long doublestrand RNA in the cytoplasm is processed into 21- to 23-nucleotide siRNAs with the characteristic 3' overhangs of two nucleotides by Dicer, a ribonuclease III enzyme. Second, the siRNA is incorporated into an RNA-induced silencing complex (RISC). Third, the RISC complex uses the guide strand (usually the antisense strand) of the siRNA to bind to its target messenger RNA (mRNA). Finally, the target mRNA is cleaved, and the expression of the target gene is inhibited. This process of siRNA inhibition is shown schematically in Figure 1.

Because carefully designed siRNA can specifically inhibit the expression of its target gene without affecting other genes, RNAi has become an effective tool for gene functional analysis [3–10]. For example,

using an RNAi library targeted to nearly 90 % of the 19,427 predicted genes of *Caenorhabditis elegans*, Simmer et al. investigated the relationship between the phenotype and related sets of genes [6]. In addition, Keating et al. explored the functions of 60 G protein-coupled receptors in *C. elegans* by RNAi on the whole-genome level [3]. In addition to gene functional analysis, RNAi has also been widely studied for genetic-based therapies. As such, RNAi is a potentially useful method to develop highly specific dsRNA-based gene-silencing therapeutics [11]. RNAi has been called 'one of the most exciting discoveries in biology in the last couple decades' [1]. However, designing siRNAs with high efficiency and high specificity is critical. Here we present a minireview about this topic.

We will briefly describe the potential applications of RNAi as an alternative therapeutic strategy, and provide a more detailed discussion of the main prediction methods for siRNA efficiency. Next, we review off-target effects analysis, and indicate some Web servers for siRNA design. Finally, we summarize the review and present possible directions for future research in developing new prediction methods for siRNA efficiency.

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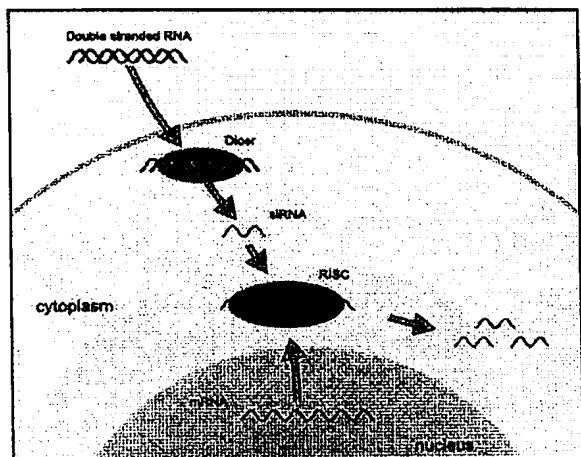


Figure 1. The main processes of short interfering RNA inhibiting expression of its target gene.

#### Potential applications of RNAi as an alternative therapeutic strategy

As an alternative therapeutic strategy, compared to conventional drugs, an RNAi-based potential drug has the following advantages [12]: easy to select target sites, high efficiency, ability to degrade cognate mRNA in cells of different species, no side effects, and stability. From a theoretical point of view, any gene whose expression contributes to disease is a potential target, from viral genes to oncogenes to genes responsible for heart disease and so on [1]. Through the design of specific siRNAs for the relevant genes, the expression of the target gene can be destroyed without affecting the expression of other genes. RNAi thus holds promise to become a therapeutic agent in the near future.

Up to now, RNAi has been widely employed as a potential therapeutic strategy in viral infections [13–28], drug target discovery [29–33], cancers [34–38], and inherited genetic disorders [39, 40]. For example, in order to inhibit the infection of human immunodeficiency virus-1 (HIV-1), Novina et al. devised five siRNAs targeted to the HIV-1 cellular receptor CD4 and the viral structural Gag protein. They found that infection with HIV-1 can be efficiently inhibited in mammalian cells [41]. Song et al. studied the application of RNAi in fulminant hepatitis. By designing six siRNAs targeted to Fas, a gene related to apoptosis in a broad spectrum of liver diseases [42], the researches found that the longevity of 82% of experimental mice could be significantly prolonged. Based on conservation among the major HBV (hepatitis B virus) genotypes [15], seven siRNA sequences targeted to HBV mRNA were designed and used to inhibit HBV

in mice by McCaffrey et al.. The results showed that six out of seven siRNAs have an antiviral effect. Two of them are very potent. In the antiviral application of RNAi, severe acute respiratory syndrome (SARS) coronavirus [26, 43], hepatitis C virus (HCV) [24], and herpes simple virus 2 (HSV-2) [28] have also been studied. For a recent review of siRNAs as the potential drugs, see [44]. For potential applications in cancer therapy, see [35, 45]. In addition, through the genome-wide RNAi screen in *Drosophila* cells infected with *Drosophila* C virus, Cherry et al. have found a related 66 specific ribosomal proteins which are required for RNA viruses with internal ribosome entry sites (IRESs) to be translated [33]. These host proteins may be potential therapeutic targets for curing the related RNA virus. For a review of its applications in drug target discovery, see [31].

From the point of view of systems biology, genes do not act independently. They interact with one another and form genetic networks. Mapping these networks plays an important role in understanding the state of biological systems, especially complex human disease [46]. In their recent paper [47], Lehner et al. developed a method for a high-throughput RNAi genetic interaction screen. In order to test the effects of the screen method, they chose the known pathways of EGF (epidermal growth factor), Wnt, Notch, SynMuvA, and SynMuv B. By considering all possible genetic interactions between ~31 genes in the above pathways and ~1750 library genes, they found 349 genetic interactions between 162 genes which function in the signaling and transcriptional networks. This is the first time RNAi technology has been used for systematic mapping of genetic interactions in *C. elegans* (animals). Through the experiments, the researches not only validated the known pathway components but also identified new pathway components. In addition, from the 349 genetic interactions, they also found six 'hub' genes, i.e., a class of highly connected genes. The hub genes are all chromatin-remodeling components. These genes can act as genetic buffers for a diverse set of genes, and therefore regulate many biological processes. Furthermore, Lehner et al. predicted that human orthologs of these genes are likely to be related to the human genetic diseases. This RNAi-based high-throughput screen method for genetic interaction may provide a way to predict complex human disease-related genes. From the above analysis, we conclude that RNAi is a powerful tool and has been applied in many aspects of molecular biology. Although there are many factors, such as target selection, delivery of siRNA, and so on, involved in the applications of RNAi, siRNA efficiency is one of the most important factors. We will discuss it in detail in following sections.

### Predicting siRNA efficiency

Because of the wide application of RNAi, many prediction methods for siRNA efficiency have been developed in recent years [48–59]. These methods are mainly based on sequence characteristics of siRNAs or target mRNA secondary structures. For each kind of prediction method, the steps to construct a mathematical model are as follows. First, a certain number of siRNA sequences and related inhibiting efficiency vis-a-vis their target genes are collected. Second, the collected siRNA sequences are classified into two groups based on inhibition efficiency (high or low efficiency group). Third, the potential characteristics related to siRNA efficiency are extracted from siRNA sequences or target mRNA secondary structures. Finally, the significant characteristics between the two groups are identified, and these characteristics can be used to guide design of siRNAs with high efficiency for new target mRNA sequences.

#### Prediction methods of siRNA efficiency based on siRNA sequence characteristics

For prediction methods based on siRNA sequence characteristics, the basic assumption is that siRNA efficiency is mainly determined by the siRNA sequence itself. Currently, many sequence characteristics-based methods have been developed. For example, using the experimental data of 180 siRNAs targeted to two target genes, Reynolds et al. presented eight rules for designing high-efficiency siRNAs [48]. These eight rules are as follows: 1. The GC content of the sense strand of siRNA is between 30 and 50 %. 2. There are more than three bases A or U in the region (15th–19th base) of the sense strand. 3. The stable hairpin structure of the sense (antisense) strand of siRNA is not permitted, and the melting temperature ( $T_m$ ) should be less than 20°C. 4. The 19th base of the sense strand of the siRNA should be A. 5. The third base of the sense strand should be A. 6. The 10th base of the sense strand should be U. 7. The 19th base of the sense strand should not be G or C. 8. The 13th base of the sense strand should not be G. Furthermore, a simple algorithm was developed for high-efficiency siRNA design using the above eight rules. For each candidate siRNA sequence, a score  $S$  is assigned with initial value  $S=0$ . If a candidate siRNA satisfies conditions 1, 3, 4, 5, or 6, then  $S$  is added one ( $S=S+1$ ). If a candidate siRNA can not meet the conditions 7 or 8, then 1 is subtracted from  $S$  ( $S=S-1$ ). For condition 2, for each base A or U in the region (15th–19th base) of the sense strand of siRNA,  $S$  is added one ( $S=S+1$ ). Finally, for each candidate siRNA, there is a corresponding score  $S$ . The larger  $S$  is, the more likely the siRNA has high efficiency. In general cases, the score  $S$  is required to be more than 6

( $S \geq 6$ ). Through an independent test of 30 siRNAs targeted to 6 genes, the results indicated that there were 17 siRNAs with inhibiting efficiency 80 %. The correct rate is 56.7 %. The shortcoming of the method is that the above eight rules are treated equally. In fact, there is no reason to suppose that the efficiency contribution of the eight rules is equal in inhibiting target mRNA.

Recently, Jagla et al. used a larger experimental dataset of 601 siRNAs targeted to 4 genes to deduce the sequence characteristics for high-efficiency siRNA design [49]. From the whole dataset, four separate training datasets were extracted. For each training set, the decision-tree classification method was used to deduce criteria for siRNAs with high efficiency. There were four sets of criteria altogether. These four sets of criteria were merged, and the significant rules were found as follows: 1. The 10th and 19th bases of the sense strand of siRNA should be A or U. 2. The first base of the sense strand should be G or C. 3. There are more than 3 bases A or U in the region (13th–19th base) of the sense strand of siRNA. Obviously, rule 1 is basically the same as Reynolds rules 6 and 7. Rule 3 contains the information of Reynolds rule 2. Finally, the results indicated that prediction accuracy is about 30 % when the inhibiting efficiency of 70 % was taken as a threshold to distinguish a high- or low-efficiency group based on an independent test dataset.

Even through the above algorithms conclude that sequence characteristics are very important in determining the efficiency of siRNAs, Patzel et al. consider that the secondary structure of siRNA antisense strand plays an important role [50]. From the experimental data of nine siRNAs targeted to one gene, they found that the siRNA efficiency would be improved if there were more free bases at the 5' or 3' end of the antisense strand. Here free bases refers to the bases that form no base pairs with other bases. The correlation between the number of free bases and siRNA efficiency was as high as 0.94. Further analysis showed that siRNA efficiency was basically irrelevant to the accessibility of target mRNA, sequence characteristics, and the 5' end stability of the antisense strand. Although the correlation between the number of free bases and siRNA efficiency is high, the number of siRNAs is too few. The above conclusions may need further verification.

In order to improve the success rate of designing high-efficiency siRNAs, Hall et al. have constructed a neural network based on the experimental data of 2182 siRNAs targeted to 34 genes [51]. First, from 2182 siRNAs, they extracted the 200 siRNAs with the highest efficiency and the 200 siRNAs with the lowest efficiency as the training dataset. Second, the neural network was constructed using the characteristics of

siRNA sequences. Finally, an independent test dataset containing 249 siRNAs was used to test the neural network. The results indicated that the Pearson coefficient between the prediction results and experimental data was 0.66. Even so, as pointed out by Miyagishi et al. in a comment, the prediction accuracy will improve further if the secondary structures of the targets are considered. Therefore, for the improvement of prediction accuracy of siRNA efficiency, more factors based on the sequence characteristics should be taken into account.

#### Prediction methods of siRNA efficiency based on secondary structures of target mRNA

The typical prediction method based on secondary structures of the target mRNA is the H-bond method, introduced by Luo et al. [53]. In order to deduce the relationship between the target secondary structures and siRNA efficiency, the H-bond index (H-b) was calculated for the siRNAs according to the following steps. First, a certain number of target secondary structures, whose free energy is close to the minimum free energy, were predicted using the Mfold Web server [60]. Second, the frequency for each base in the target region paired to other bases was calculated. For example, if the number of the calculated target secondary structures is 50, and there are 30 opportunities for the first base in the target region to be paired with other bases, the frequency is 0.6 (30/50). Furthermore, if the base in the target region is G or C, the corresponding frequency is multiplied by 3. If the base in the target region is A or U, the corresponding frequency is multiplied by 2. Finally, H-b is calculated by summing of all the weight frequencies in the target region. Based on H-b, the quantitative relationship between siRNA efficiency and target secondary structure was observed using an experimental dataset of 14 siRNAs targeted to 3 genes. The results indicated that siRNAs with high efficiency had a low H-b. Finally, according to the above conclusions, four siRNAs targeted to a new target gene were designed. The experimental results further confirmed the conclusions. Obviously, if the target region is in the single-chain state, H-b will be very low. From this point of view, siRNAs with high efficiency should be single chains. This conclusion was also observed by Schramm et al. [54]. In addition, Overhoff et al. confirmed that siRNA efficiency should be high if the target region was accessible in the target mRNA [55]. Recently, Yiu et al. presented a repelling loop method to evaluate siRNA efficiency based on five minimum or near-minimum free-energy target mRNA secondary structures [56].

There are three challenges in analyzing the relationship between siRNA efficiency and target mRNA secondary structures. First, the most popular method for RNA secondary structure prediction at present is

Zuker's Mfold Web server. The prediction accuracy is low for long target mRNA sequences. However, the target mRNA usually is long. For example, from the siRNA database siRecord [61] we obtained 1354 target mRNA sequences without missing values. Among 1354 targets, the shortest and longest sequence lengths are 51 and 2,314,078 bases, respectively. The average target sequence length is 5877 bases, which makes it difficult to get accurate target mRNA secondary structures. Therefore, reliable results of analysis based on target secondary structures cannot be guaranteed. Second, for long target mRNA sequences, the computation time is long and the demands on the computer are high. Sometimes, current PC's struggle to meet the requirements of computation. Third, although Luo et al. formulated the H-b method to analyze the relationship between siRNA efficiency and secondary structures of target mRNA quantitatively [53], we still do not know how many target secondary structures need to be calculated. Furthermore, the number of samples used in Luo's analysis is very small, which makes it difficult to guarantee the reliability of H-b.

#### Analysis of off-target effects

For particular target mRNAs, successful design of siRNAs requires not only high efficiency to silence the target gene but also high specificity. Here, specificity means that the designed siRNAs inhibit only the expression of the target gene, i.e., the designed siRNAs should not have off-target effects. Analysis of off-target effects plays an important role in applications of RNAi. For example, siRNAs may have potential as drugs for treatment of viral diseases. If the designed siRNAs have off-target effects on the human genes, side-effects will result.

In order to avoid off-target effects, many methods and programs have been developed [62–67]. However, the key issue is what criteria should be used to analyze these effects. At first, people found that silence effects can be abolished by only a single central mismatch between the siRNA and corresponding target mRNA [68]. Therefore, the blast program was used to check specificity [69]. The process is very similar to the probe specificity check for oligonucleotide microarrays [70]. First, related parameters such as word length and E value for the blast program are set up. In order to avoid potential other targets of the designed siRNAs, word length is often assigned as the minimum value. Second, the related transcriptome database is selected for the siRNAs. For example, if the target mRNA is a human gene, then the human transcriptome database should be selected. Better siRNAs are

**Table 1.** Selected Web servers for siRNA design.

Author(s)	Web page	Ref.
Schramm et al.	<a href="http://www.mwg-biotech.com">http://www.mwg-biotech.com</a>	54
Naito et al.	<a href="http://design.rnai.jp/">http://design.rnai.jp/</a>	75
Henschel et al.	<a href="http://cluster-1.mpi-cbg.de/Deqor/deqor.html">http://cluster-1.mpi-cbg.de/Deqor/deqor.html</a>	76
Cui et al.	<a href="http://bioit.dbi.udel.edu/rnai/">http://bioit.dbi.udel.edu/rnai/</a>	77
Dudek et al.	<a href="http://www.cellbio.unige.ch/RNAi.html">http://www.cellbio.unige.ch/RNAi.html</a>	78
Arziman et al.	<a href="http://www.dkfz.de/signaling2/e-rnai/">http://www.dkfz.de/signaling2/e-rnai/</a>	79
Yuan et al.	<a href="http://jura.wi.mit.edu/bioc/siRNAext/">http://jura.wi.mit.edu/bioc/siRNAext/</a>	80
Wang et al.	<a href="http://www.genscript.com/sirna_ca.html#design">http://www.genscript.com/sirna_ca.html#design</a>	81
Levenkova, et al.	<a href="http://bioinfo.wistar.upenn.edu/siRNA/siRNA.htm">http://bioinfo.wistar.upenn.edu/siRNA/siRNA.htm</a>	82
Santoyo et al.	<a href="http://side.bioinfo.cnio.es">http://side.bioinfo.cnio.es</a>	83

found based on analysis of the blast results. In view of their fast running speed, blast programs are still used in the design of siRNAs.

However, people also found that the siRNAs could tolerate several mismatches between the siRNAs and the target. Findings show that blast programs may be not suitable for specificity analysis of siRNAs because blast programs can miss some potential targets, which leads to off-target effects. For example, in an investigation of 359 published siRNA sequences, Snove et al found that about 75 % of them had a risk of eliciting non-specific effects [71]. New programs have been developed based on these findings.

In Order to deduce the criteria for specificity analysis of siRNAs, gene expression profiling regulated by RNAi was used. Jackson et al. concluded that the similarity between siRNA and target mRNA is 79 % (15/19) and perhaps as few as 11 contiguous nucleotides between them are sufficient to direct silencing of nontargeted transcripts [72]. These results are very similar to Kane's criteria for probe design for oligonucleotide microarrays [73].

Recently, the criteria for off-target gene silencing have been studied in detail using gene expression profiling of human cells transfected with siRNAs [74]. From the expression profile regulated by 12 siRNAs, Birmingham et al. identified 347 off-target genes as the experimental off-targets set. The Smith-Waterman algorithm was used to calculate the similarity between the siRNAs and human genes, and the *in silico* off-targets set was obtained with similarity more than 79 %. Comparison of the experimental off-targets set and the *in silico* off-targets set showed that the false positive rate and false negative rate were more than 99 % and 93 %, respectively. In the meantime, the researches found that off-target effects were associated with the presence of one or more perfect matches (6–7 bases) between the antisense strand of the siRNA and the 3' untranslated region of the off-targets. These results

showed that off-target effects were mainly caused by 3' UTR seed matches rather than the overall identity between siRNAs and non-target mRNAs.

In future, to determine the criteria for siRNA specificity checks, we may need further experiments. Also, the secondary structures of nontarget mRNAs and the potential target regions of nontarget mRNAs should be considered.

#### Software for highly efficient siRNA design

The point of predicting siRNA efficiency and analysis of off-target effects is to design siRNAs with high efficiency and high specificity for further experiments. Therefore, it is very important to develop software for automatic design of siRNAs. Many programs have been developed. Some of them are listed in Table 1 [75–83]. In view of the long running time needed to predict the secondary structures of target mRNA, the programs are basically based on the methods of siRNA sequence characteristics.

#### Conclusions

From above analysis, we conclude that there are many factors involved in siRNA efficiency. These factors include secondary structures of the sense and antisense strands of siRNAs, secondary structures of target mRNAs, sequence characteristics of siRNAs, and the specificity of siRNA to its target. The analysis of siRNA efficiency, poses three problems at present. The first is the relationship between siRNA efficiency and the secondary structures of target mRNAs. Even though some point out that there is a strong relationship between them, others think that the siRNA efficiency is mainly determined by siRNA itself. The second problem is that current analysis of



siRNA efficiency is mainly based on factors such as the secondary structure of siRNA antisense strands rather than comprehensive analysis of all factors. The third problem is the off-target analysis of siRNA. Even though some criteria have been formulated, there is no standard for siRNA specificity. In order to design siRNAs with high efficiency and high specificity, all these problems should be considered comprehensively, and new algorithms should be developed. To meet this objectives, both supervised learning methods and optimal feature selection procedures may be needed.

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1792 W. Li and L. Cha

Predicting siRNA efficiency

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